Evidence Appendix

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Anti-atherosclerotic effect of simvastatin depends on the presence of apolipoprotein E

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Abstract

Low density lipoprotein receptor deficient (LDLR-KO) and apolipoprotein E deficient (apo E-KO) mice both develop hyperlipidemia and atherosclerosis by different mechanisms. The aim of the present study was to compare the effects of simvastatin on cholesterol levels, endothelial dysfunction, and aortic lesions in these two models of experimental atherosclerosis. Male LDLR-KO mice fed a high cholesterol (HC; 1%) diet developed atherosclerosis at 8 months of age with hypercholesterolemia. The addition of simvastatin (300 mg/kg daily) to the HC diet for 2 more months lowered total cholesterol levels by ~57% and reduced aortic plaque area by ~15% compared with the LDLR-KO mice continued on HC diet alone, $P < 0.05$. Simvastatin treatment also improved acetylcholine (ACh)-induced endothelium-dependent vasorelaxation in isolated aortic rings, which was associated with an increase in NOS-3 expression by ~88% in the aorta measured by real time polymerase chain reaction (PCR), $P < 0.05$. In contrast, in age-matched male apo E-KO mice fed a normal diet, the same treatment of simvastatin elevated serum total cholesterol by ~35%, increased aortic plaque area by ~15%, and had no effect on endothelial function. These results suggest that the therapeutic effects of simvastatin may depend on the presence of a functional apolipoprotein E.

Keywords: HMG-CoA reductase inhibitor; Atherosclerotic plaque; Cholesterol; Endothelial dysfunction; NOS-3; Apo E-knockout; LDLR-knockout; Mouse

1. Introduction

Hypercholesterolemia is a major risk factor for development of atherosclerotic vascular disease [1]. Hydroxy-methylglutaryl-coenzyme A (HMG CoA) reductase inhibitors (statins) lower cholesterol and reduce cardiovascular morbidity and mortality in patients with atherosclerosis [2–4]. A growing data base suggests that the beneficial actions of statins may be due to direct effects on the vascular wall in addition to lipid lowering. Moreover, atherosclerosis is often accompanied by endothelial dysfunction due to impaired endothelial nitric oxide (NO) production [5–8]. Statins have been shown to restore endothelia function by restoring NO-mediated vasodilation in hyperlipidemic rabbits [9] and in patients with coronary artery disease [10,11]. This improvement in endothelial function contributes to the cardiovascular benefits achieved by statin treatment.

Low density lipoprotein receptor deficient (LDLR-KO) and apolipoprotein E deficient (apo E-KO) mice have been used to study mechanisms of atherogenesis [12,13]. It has been shown that simvastatin lowers lipid levels in LDLR-KO [14], but not in apo E-KO [15] mice. The present study was to compare the effect of simvastatin on atherosclerosis development be-

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tween the two animal models of atherosclerosis. In addition, the effects of simvastatin on endothelial function and endothelial nitric oxide synthase (NOS-3) were also examined.

2. Methods

2.1. Animals and experimental design

Two-month-old male LDLR-KO mice (Jackson Laboratories, Bar Harbor, ME) were fed a high cholesterol (HC; 1%) diet for 8 months. At 10 months of age the animals developed moderate atherosclerotic lesions in the aorta ($35 \pm 3\%$) accompanied by hypercholesterolemia (591 ± 75 mg/dl). They were then randomly divided into three groups, control group; continued on a HC diet; simvastatin group; fed a HC diet supplemented with 0.15% simvastatin (HC + SIM); and regular diet (RD) group, withdrawn from the HC diet and fed a regular chow diet. The above treatments were continued for 2 months.

Apo E-KO mice spontaneously develop hypercholesterolemia and atherosclerosis without the need for a cholesterol supplementation. For the present studies, 10-month-old male apo E-KO mice (Jackson Laboratories, Bar Harbor, ME) were used. One group of mice were fed a grain-based rodent diet (Bio-Serv, NJ) as controls and the other was on the same diet supplemented with 0.15% simvastatin for 1–3 months. The daily dose of simvastatin in both LDLR-KO and apo E mice was approximately 300 mg/kg, which has been shown to effectively reduce cholesterol by 37% in LDLR-KO mice [14].

At the end of the treatment period, all animals were fasted overnight and euthanized. Blood samples were collected via cardiac puncture at the time of death. Total serum cholesterol, triglycerides and high density lipoprotein (HDL) levels were determined enzymatically (performed by IDEXX, West Sacramento, CA). LDL values were calculated from total cholesterol and HDL levels. The aortae were isolated for measurements of atherosclerotic lesion area, vascular reactivity, and NOS-3 mRNA expression.

2.2. Measurement of atherosclerotic plaque

The aortae were isolated, cleaned from the adherent connective tissue, fixed with 10% formalin, cut open longitudinally and pinned on black wax-coated petri dishes as previously described in detail [5]. Atherosclerotic plaque area is visible without staining. The images of the open luminal surface of the aortae were recorded at a resolution of 512×512 using a RGB 3-chip CCD digital camera (Sony) mounted on a dissecting microscope (Nikon SMZ-2T) attached to a computer in 24 bit true image format. The images were analyzed using C-Simple software (C. Imaging 1208, Compix, Mars, PA). Atherosclerotic plaque area was quantified and expressed as a percentage of total luminal surface area of the aorta.

2.3. Assessment of vascular reactivity

The thoracic aortae were dissected, cleaned from the adherent connective tissue, and placed in a HEPES-buffered solution containing (in mM), 140 NaCl; 4.5 KCl; 1.0 $MgCl_2$; 5.5 glucose; 1.5 $CaCl_2$; and 10 HEPES at pH 7.4 and 20 °C. The aortae were cut into four rings and were placed in organ-bath chambers containing 15 ml of Krebs solution with the following composition (in mM), 118 NaCl; 24.9 $NaHCO_3$; 4.7 KCl; 1.18 KH_2PO_4; 1.66 $MgSO_4$; 5.55 glucose; 2.0 Na-pyruvate; and 2.0 $CaCl_2$. The solution was continually bubbled with a 5% CO_2 and 95% O_2 gas mixture and maintained at pH 7.4 and 37 °C. Vessels were pre-treated with indomethacin (10^{-5} M) for 30 min to inhibit cyclooxygenase mediated vascular effects and pre-contracted with KCl (40 mM), and washed with Krebs solution. Aortic rings were then stretched to 500 mg tension and allowed to equilibrate for 2 h prior to initiation of the experimental protocol. Tension measurements were recorded using Grass force-transducers connected to a data acquisition system (MP100 WS, Biopac, Goleta, CA). Data were digitized on-line at a rate of 1 sample per s and subsequently analyzed using Acknowledge software. Concentration response curves to U46619 (9,11-dideoxy-9α, 11α-methanoepoxy prostaglandin $F_{2\alpha}$), a thromboxane receptor agonist, were then generated. The calculated concentration of U46619 that produced 80% of the maximal contractile response (EC_{80}) was 20 nM. Endothelium-mediated relaxation was measured as the response to acetylcholine (Ach; 0.01 nM–10 μM) in rings pre-contracted with U-46619 (30 nM). In the LDLR-KO mice, endothelium-independent aortic ring relaxation was also measured as the response to sodium nitroprusside (SNP, 0.001–1 μM).

2.4. Measurement of NOS-3 mRNA

The isolated aortae were homogenized in 600 μl RLT buffer (Qiagen) using disposable generator probes (Omni International). Total RNA was then isolated using a RNeasy kit with DNase I digestion (Qiagen). Relative abundance of NOS-3 and internal control GAPDH were measured by real-time quantitative polymerase chain reaction (PCR) performed on an ABI PRISM 7700 Sequence Detector (PE Biosys-

tems). One-step reverse transcriptase (RT)-PCR amplification of NOS-3 was carried out in a 50 μl reaction mixture consisting of 1 × TaqMan buffer A with the following composition (mM), 5.5 $MgCl_2$; 0.3 dATP; 0.3 dCTP; 0.3 μM dGTP; 0.3 dUTP; 0.025 U/μl AmpliTaq Gold DNA polymerase, 0.025 U/μl RNase inhibitor, 0.025 U/μl multiscribe RT, 200 nM of each primer, and 100 nM probe. Thermal cycle conditions were 48 °C for 30 min and 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Primers and the probe for NOS-3 were, upper primer, 5′-CGTCATCGGCGTGCT-3′ (nt 3436–3450), lower primer, 5′-ACCTCCTGGGTGCGC-3′ (nt 3510–3496), and the probe, 5′-6FAM -CGGGATCAGCAACGCTACCA-TAMRA-3′ (nt 3452–3471). Primers and TaqMan probe for rodent GAPDH were purchased from PE Biosystems (P/N 4308313). Hundred nanomol of each primer and 200 mM of the probe were used in the reaction. The expressions of NOS-3 and GAPDH were calculated against a standard curve with serial dilution of total RNA from murine hemangioendothelioma (EOMA) cells [16]. The experiment was repeated twice in triplicate for each sample. Values presented here are the ratio of NOS-3/GAPDH.

2.5. Statistics

All results are presented as the mean ± S.E.M. for the number of animals (n) indicated. Multiple comparisons of mean values were performed by analysis of variance (ANOVA) followed by a subsequent Student–Newman–Keuls test for repeated measures. Differences were considered to be statistically significant when the P value was < 0.05. The statistical analysis was performed using Statistica software (STATSOFT, Tulsa, OK).

3. Results

3.1. Effects of simvastatin in LDLR-KO mice

LDLR-KO mice fed a HC diet for 10 months had hypercholesterolemia and developed atherosclerotic lesions in the aorta (Fig. 1). Treatment with simvastatin (HC + SIM) decreased serum LDL cholesterol with no significant effects on HDL cholesterol or triglycerides levels (Table 1). As a result, the ratio of HDL/LDL was significantly higher in the HC + SIM compared with the HC group (Table 1). Simvastatin also reduced atherosclerotic lesion area by ~15%, compared with that in the HC group (Fig. 1). Mice given the regression diet for 2 months showed similar changes in lipid profiles and atherosclerotic lesions as were seen following simvastatin treatment (Table 1 and Fig. 1). Combining the data from all three groups, aortic atherosclerotic lesion area was positively correlated to total serum cholesterol levels and negatively correlated to the ratio of HDL/LDL (Fig. 2).

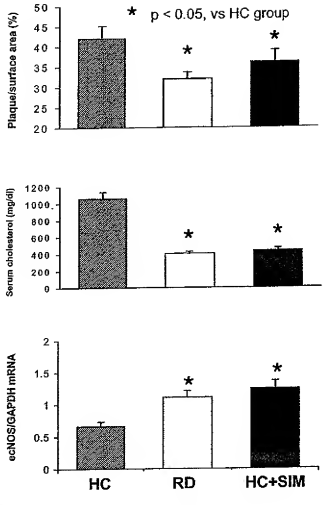


Fig. 1. Aortic atherosclerotic lesion area (top, $n = 8$ per group), total serum cholesterol levels (middle, $n = 11$ per group), and NOS-3 mRNA expression in the aorta (bottom, $n = 3$ per group) of the LDLR-KO mice fed a HC diet without or with (HC + SIM) the supplementation of simvastatin (300 mg/kg, daily), or withdrawn from a HC diet and fed a RD, for 2 months.

Table 1
Effects of simvastatin and diet on serum lipid profile (mg/dl) in LDLR-KO mice ($n = 11$ per group)

	HC	RD	HC + SIM
LDL	917 ± 80	256 ± 19**	322 ± 27**
HDL	98 ± 6	102 ± 8	77 ± 6
HDL/LDL	0.12 ± 0.01	0.38 ± 0.04**	0.24 ± 0.03**
Triglyceride	213 ± 16	226 ± 19	175 ± 21

*, P, 0.05; **, $P < 0.01$; vs. HC.

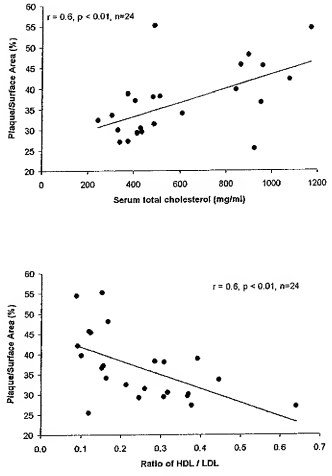


Fig. 2. The aortic atherosclerotic lesion area was positively correlated to the total circulating cholesterol levels (top) and negatively correlated to the ratio of HDL/LDL in the LDLR-KO mice. The data included all three groups of the mice fed a HC diet without or with (HC + SIM) the supplementation of simvastatin (300 mg/kg, daily), or withdrawn from a HC diet and fed a RD, for 2 months.

ACh-induced endothelial NO-mediated aortic relaxation was significantly greater in both the HC + SIM and RD groups than that in the HC group (Fig. 3A). Thus, the maximum responses were significantly greater in both the HC + SIM and RD groups than that in the HC group (Table 2). Endothelium-independent relaxation to SNP did not significantly differ among the three groups (Fig. 3B and Table 3). The expression of NOS-3 mRNA was significantly higher in both the HC + SIM and RD groups than the expression levels in the HC group (Fig. 1).

3.2. Effects of simvastatin in apo E-KO mice

Age-matched apo E-KO mice fed a RD had hypercholesterolemia and developed atherosclerotic lesions in the aorta, which tended to increase over time (Fig. 4). Serum total and LDL cholesterol levels were higher and HDL cholesterol levels lower in the simvastatin than in the control group (Fig. 4 and Table 3). As a result of these changes, the ratio of HDL/LDL was significantly lower in the simvastatin than the control group. Triglyceride levels were not significantly different between the two groups. Aortic lesion area was greater in the simvastatin than control group (Fig. 4). Combining the data from both groups at all time points, aortic atherosclerotic lesion area was positively correlated to total serum cholesterol levels and negatively correlated to the ratio of HDL/LDL (Fig. 5).

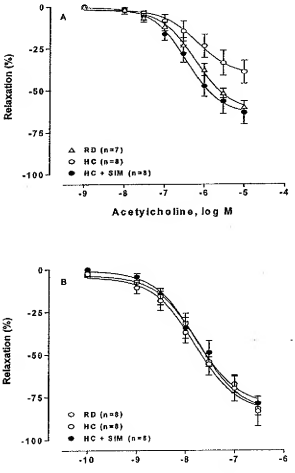


Fig. 3. Concentration–response curves of ACh (A) or SNP (B) induced relaxation in the aortic rings isolated from LDLR-KO mice fed a HC diet without or with (HC + SIM) the supplementation of simvastatin (300 mg/kg, daily), or withdrawn from a HC diet and fed a RD, for 2 months.

Table 2
Sensitivity (log EC_{50}, pD_2) and maximal response (E_{max}) to ACh and SNP in isolated aortae from LDLR-KO mice fed a RD, HC diet without or with simvastatin (HC+SIM)

Groups	N	ACh		SNP	
		pD_2	E_{max} (%)	pD_2	E_{max} (%)
HC	8	6.17 ± 0.20	39.2 ± 6.8*	7.74 ± 0.18	81.8 ± 3.8
RD	7	6.2 ± 0.16	60.2 ± 3.6	7.8 ± 0.21	83.3 ± 8.5
HC+SIM	8	6.45 ± 0.11	63.4 ± 6.9	7.79 ± 0.22	78.8 ± 4.2

* $P<0.05$ vs. RD group.

ACh-induced relaxation of the aortae isolated from apo E-KO mice were not significantly different between the simvastatin and control groups at both the 2 and 3 months time points (Fig. 6 and Table 4).

4. Discussion

The major findings of the present study are that simvastatin has opposite effects on serum lipids and atherosclerosis in two different genetic mouse model of atherosclerosis. In the LDLR-KO mice, simvastatin decreased serum cholesterol levels and aortic lesion area. Theses changes were associated with an improvement in endothelial NO-dependent vasorelaxation and an increased NOS-3 mRNA expression. In contrast, in the apo E-KO mice, the same treatment with simvastatin increased serum cholesterol levels and aortic lesion area, with no changes in endothelial NO-mediated vasorelaxation.

Table 3
Effects of simvastatin on serum lipid profiles (mg/dl) in apo E-KO mice ($n = 7$–12 per group)

Treatment (month)	Vehicle	Simvastatin
*LDL***		
1	463 ± 55	630 ± 41
2	474 ± 42	727 ± 39
3	462 ± 46	605 ± 42
*HDL**		
1	70 ± 6	59 ± 4
2	88 ± 10	51 ± 6
3	100 ± 5	48 ± 6
*HDL/LDL**		
1	0.16 ± 0.01	0.10 ± 0.01
2	0.19 ± 0.02	0.07 ± 0.01
3	0.26 ± 0.05	0.08 ± 0.01
Triglycerides		
1	193 ± 32	198 ± 48
2	173 ± 19	141 ± 7
3	158 ± 21	109 ± 8

*, $P<0.05$; **, $P<0.01$ between two groups.

These data indicate that an intact functional apolipoprotein E may be essential for the lipid lowering, anti-atherosclerosis and other therapeutic benefits of simvastatin.

4.1. Effects of simvastatin on hypercholesterolemia

In the present study, simvastatin at a daily dose of 300 mg/kg significantly reduced total cholesterol by

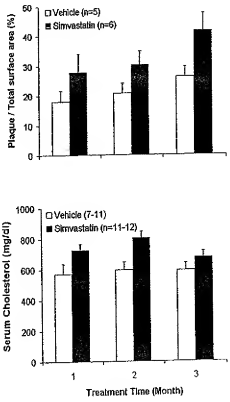
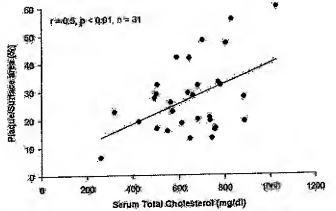


Fig. 4. Atherosclerotic lesion area in the aorta (top) and total serum cholesterol levels (bottom) of the apo E-KO mice treated with simvastatin (300 mg/kg, daily) or vehicle for 1–3 months. $P<0.05$ between the simvastatin and vehicle treatment group for both the plaque area and total serum cholesterol levels.

57% in LDLR-KO mice fed a HC diet. This result is consistent with a previous report by Bisgaier et al., who reported that at the same daily dose, simvastatin reduced total circulating cholesterol by 37% in LDLR-KO mice [14]. The magnitude of cholesterol lowering by simvastatin was also similar to that observed in untreated LDLR-KO mice fed a regression diet for 2 months. In contrast, the same dose of simvastatin resulted in a 27% increase in serum cholesterol in apo E-KO mice. This finding is consistent with that of Quarfordt et al., who also reported that lovastatin (50 mg/kg daily) increased circulating cholesterol by 70% in apo E-KO mice, but not in wild-type controls [15]. These results suggest that the lipid lowering effect of statins may depend on the presence of intact apolipoprotein E, which functions to transport circulating cholesterol into cells, particularly hepatocytes and acts as an important mediator



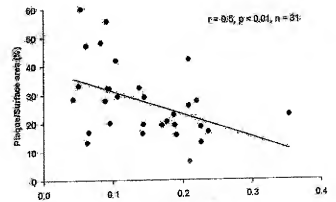


Fig. 5. The aortic atherosclerotic lesion area was positively correlated to the total circulating cholesterol levels (top) and negatively correlated to the ratio of HDL/LDL in the apo E-KO mice. The data included all groups of mice treated with simvastatin (300 mg/kg, daily) or vehicle for 1–3 months.

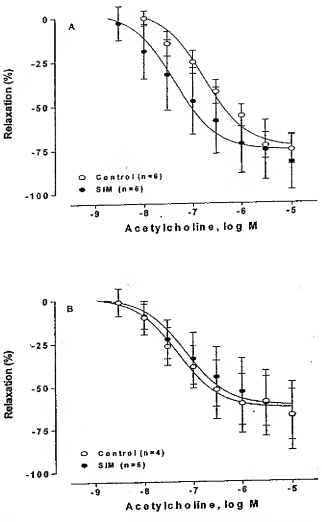


Fig. 6. Concentration–response curves of ACh-induced relaxation of the aortic rings isolated from apo E-KO mice treated with simvastatin (300 mg/kg per day) or vehicle for 2 (top) and 3 (bottom) months.

for hepatic metabolic clearance of circulating cholesterol [12]. In the absence of the apolipoprotein E, hepatic clearance and metabolism of cholesterol are reduced, resulting in hypercholesterolemia [12,13,17]. Impairment of hepatic cholesterol transport may also result in up-regulation of cholesterol synthesis [18]. Indeed, lovastatin has been reported to increase the expression of HMG CoA reductase mRNA [19] and protein [20], thereby increasing cholesterol synthesis [15] in apo E-KO mice, but not in wild-type controls. This may explain the paradoxical elevation of cholesterol in simvastatin-treated apo E-KO mice.

4.2. Effects of simvastatin on atherosclerosis

Accompanied by its lipid lowering effect, simvastatin significantly reduced aortic atherosclerotic

Table 4
Sensitivity (log EC_{150} pD_2) and maximal response (E_{max}) to ACh and SNP in isolated aortae from apo E-KO mice treated without (control) or with simvastatin for 2 or 3 months

Groups	Duration					
	Two months			Three months		
	N	pD_2	E_{max} (%)	*N*	pD_2	E_{max} (%)
Control	6	6.75 ± 0.27	75.8 ± 8.2	4	7.31 ± 0.20	67.5 ± 14.7
Simvastatin	6	7.37 ± 0.34	82.6 ± 15.7	6	7.15 ± 0.32	68.0 ± 19.8

plaque area in LDLR-KO mice. In contrast, simvastatin accelerated atherosclerosis that accompanied an elevation of serum cholesterol levels in apo E-KO mice. Hypercholesterolemia is an important risk factor leading to atherosclerosis. Animals with hypercholesterolemia, resulting from either a HC diet [21–23] or from inherited defects in lipid metabolism [5–7], develop atherosclerotic plaques in the vascular wall. Therefore, the reduction in atherosclerotic lesions by simvastatin in LDLR-KO mice can be explained by its lipid lowering effect, since similar reduction in serum cholesterol and aortic lesion area were observed in untreated mice fed a RD. On the other hand, the increase in atherosclerosis by simvastatin in apo E-KO mice could be explained by elevated serum lipids. This is consistent with a previous report that feeding a HC diet to apo E-KO mice further exacerbated hypercholesterolemia and accelerated lesion development [24]. HDL is known to be involved in reversing cholesterol transport from the vascular wall [25,26]. Therefore, elevation of HDL/LDL ratio by simvastatin in LDLR-KO mice could also contribute to its anti-atherosclerotic effect. Likewise, reduction of HDL/LDL ratio by simvastatin in apo E-KO mice could contribute to acceleration of atherosclerosis. The fact that aortic lesions correlated positively to the total serum cholesterol levels and negatively to the HDL/LDL ratio in both the LDLR-KO and apo E-KO mice support the above view.

4.3. Effect of simvastatin on endothelial function

Endothelial dysfunction, characterized by reduced NO-dependent vascular relaxation, is an early marker of atherosclerosis [27]. Endothelial function is impaired in a number of experimental models of atherosclerosis, including hyperlipidemic rabbits [9] and apo E-KO mice [5], as well as in patients with atherosclerosis [8]. Statins have been shown to reverse endothelial dysfunction in hyperlipidemic rabbits [9] and in humans [10,11]. This effect of statins has been attributed to stabilization of NOS-3 mRNA leading to the increase in NOS-3 expression [28,29]. Indeed, in the present study, simvastatin treatment significantly increased the expression of NOS-3 mRNA in the aorta of LDLR-KO mice. This was associated with enhanced ACh-induced endothelial NO dependent vasorelaxation. Although there is strong evidence that direct effect on NOS-3 expression is the underlying mechanism for the improvement in endothelial function, the present study was not designed to determine the mechanism of action of simvastatin on NOS-3 and can not differentiate direct effects on the vascular wall from secondary effects due to changes in serum lipids. In apo E-KO mice, on the other hand, the potential direct beneficial effect of simvastatin on endothelial function could be masked or compromised by increased cholesterol levels and atherosclerosis. This may explain the lack of significant effect of simvastatin treatment on ACh-induced NO-dependent aortic ring relaxation in the apo E-KO mice.

During the preparation of this manuscript, Sparrow et al. published a paper demonstrating both anti-inflammatory and anti-atherosclerotic activities of simvastatin in apo E-KO mice [30]. In that study, simvastatin had no significant effect on circulating cholesterol levels. The apo E-KO mice were fed a HC diet, which resulted in a very high circulating cholesterol levels (~1000 mg/dl) and severe atherosclerosis in the aorta. The apo E-KO mice in the present study were fed a RD, resulting in moderate hyperlipidemia with serum cholesterol levels at ~500 mg/dl and a less severe atherosclerosis. These differences in the diet and circulating lipid levels, as well as the severity of atherosclerosis, may contribute, at least in part, to the different results.

In summary, the present study demonstrates that in LDLR-KO mice, treatment with simvastatin for 2 months significantly decreased serum cholesterol levels, improved endothelial function, and reduced atherosclerosis. In contrast, in apo E-KO mice, the same treatment of simvastatin elevated serum cholesterol levels and increased atherosclerosis with no effect on endothelial function. Thus, the beneficial

effects of statins may depend on the presence of functional apolipoprotein E. It has to be pointed out that the mechanisms involved in atherosclerosis induction in these animal models are different. In LDLR-KO mice, it is diet-induced, while in apo E-KO mice, it is primarily due to 'genetics'. This difference may also explain the different effects observed regarding endothelial function. Thus, whether the current findings can be applied to human, as they are less dependent on apo E for the catabolism of LDL, is a new pharmacogenetic topic that needs to be further studied.

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